Brief Definitive Report

BONE MARROW TRANSPLANTATION ACROSS MAJOR HISTOCOMPATIBILITY BARRIERS

V. Protection of Mice from Lethal Graft-vs.-Host Disease by Pretreatment of Donor Cells with Monoclonal Anti-Thy-1.2 Coupled to the Toxin Ricin*

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A major impediment to successful bone marrow transplantation is the presence of immunocompetent T lymphocytes in the donor graft which lead to the development of graft-vs.-host disease (GVHD). All too frequently, the final outcome of severe GVHD episodes is high morbidity and death. This paper represents the fifth in a series in which we search for reagents that have the requisite selectivity and consistency to eliminate GVHD. In mice GVHD can be overcome by treating marrow with complement-fixing antibodies directed toward Thy-1-expressing cells (1, 2). In a previous report we showed that treatment of donor lymphohematopoietic grafts with monoclonal anti-Thy-1.2 plus complement (C) (3) or monoclonal anti-Lyt-1 plus C (4) was effective in eliminating GVHD from allogeneic recipient mice. The use of homogeneous monoclonal antibody for this purpose is a major advance because the reagent is uniquely defined, can be reproduced at will, and does not require adsorption protocols, which are difficult to standardize. Potential complications are presented by the use of complement which must function in vivo or be supplied during in vitro pretreatment. Most notably, complement from nonhuman sources is frequently toxic for human bone marrow stem cells. We now report an alternative method for freeing donor bone marrow grafts of T cells by treating the marrow with a hybrid protein formed by the covalent conjugation of monoclonal anti-Thy-1.2 to the toxin ricin.

Ricin contains two disulfide linked subunits; the A chain inhibits ribosomal protein synthesis, and the B chain binds the protein to ubiquitous galactose-containing cell surface receptors. The native binding specificity of ricin can be modified to that dictated by covalently bound monoclonal anti-Thy-1.2 after competive blocking of the galactose-binding site on the B chain (5). The resulting protein hybrid is selectively toxic in vitro for Thy-1.2-bearing EL4 tumor cells.

In the present study, we have shown that intact ricin protein coupled to monoclonal anti-Thy-1.2 can selectively block in vitro cell function. Additionally, pretreatment of donor cells with antibody-ricin and lactose before bone marrow transplantation across major histocompatibility barriers, protected irradiated recipients from lethal GVHD.

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Materials and Methods

Hybrid Synthesis and Purification. A description of synthesis of the anti-Thy-1.2-ricin molecule has been reported (5). Briefly, ricin is reacted with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester to link maleimide residues to ricin. A standardized monoclonal IgG against Thy-1.2 (clone 30-H12 derived by Ledbetter and Herzenberg [6]) was prepared for cross-linking by partial reduction of disulfide bonds. The maleimide-linked ricin was mixed with the sulfhydryl containing antibody, forming a nonreducible thioether linkage between the two species.

Mice. C57BL/6 and BALB/c strains used in these experiments were bred and housed at the Minnesota Mouse Colony, University of Minnesota. Adult males (26–31 g) were housed in conventional cages with filter lids. Bedding, food, and water were not sterilized. Transplanted animals were fed a fat-supplemented diet and antibiotic supplemented water ad libitum.

Mitogenesis. Spleens were isolated in phosphate-buffered saline (PBS), passed through a nylon filter, and pelleted. The cells were resuspended in culture medium (Dulbeccos modified Eagle's medium plus sodium pyruvate, Hepes buffer, L-arginine, nonessential amino acids, folic acid and asparagine, L-glutamine, 2-mercaptoethanol, and 2% pooled human sera) and plated so that 5×10^5 cells were pipetted into 96-well flat-bottomed culture plates (Linbro Chemical Co., Hamden, CT) in 0.2-ml vol. To activate B lymphocytes, 25 μg/well lipopolysaccharide (LPS) from *Escherichia coli* 0111 (Difco Laboratories, Detroit, MI) was added. T lymphocytes were stimulated by the addition of 0.1 μg/well phytohemagglutinin (PHA) (Difco Laboratories). Cultures were pulsed with 1 μCi tritiated thymidine (specific activity 15 Ci/mmol) 24 h before harvest on day 3. Cultures were collected by precipitation onto glass fiber filters with an automated harvester, and thymidine incorporation was measured in a Beckman LS300 scintillation counter (Beckman Instruments Inc., Fullerton, CA) by standard counting methods.

Data were presented as percent control response and calculated as:

$$\text{percent control} = \frac{\begin{array}{r}(\text{CPM mitogen-stimulated lymphocytes + anti-Thy-1.2-ricin}) \\ - (\text{CPM lymphocytes + anti-Thy-1.2-ricin})\end{array}}{\begin{array}{r}(\text{CPM mitogen-stimulated lymphocytes untreated}) \\ - (\text{CPM lymphocytes untreated})\end{array}} \times 100.$$

Total-body Irradiation. Irradiation was delivered from a 220 kV x-ray source (General Electric Maximar-20, General Electric Co., Wilmington, MA) at 15 mA through 0.25-mm copper and 1.0-mm aluminum filters. Animals were positioned in lucite radiation chambers and exposed at a dose-rate of 50 rad/min to a total of 900 rad.

Transplantation. C57BL/6 mice were transplanted with BALB/c bone marrow plus spleen cells (BMS) as described in a previous report (3). 25×10^6 donor splenocytes were combined with an equal number of bone marrow cells and injected intravenously in 0.5 ml media.

Pretreatment of Donor Cells. Donor BMS cells were treated with monoclonal anti-Thy-1.2-ricin before injection into irradiated recipients. Based on in vitro studies, cells were adjusted to 10^7/ml in RPMI 1640, 200 mM lactose (Difco Laboratories) and preincubated at 37°C for 3 h with 0.5 μg/ml hybrid. Cells were then washed three times in media containing 100 mM lactose and resuspended for injection. Another group of recipients were given donor cells pretreated with 0.5 μg/ml antibody alone. For mitogen assays, anti-Thy-1.2-ricin and lactose were added directly to culture wells and remained throughout the assays.

Assay for Chimerism. Survival of bone marrow allografts was measured by the presence of donor-type mononuclear cells in the peripheral blood. Donor cells were detected using H-2-specific antisera in an in vitro complement-dependent microcytotoxicity assay, which has been previously described (7).

Assessment of GVHD. Following injection of treated or untreated BMS, animals were observed for the development of secondary disease. Autopsies revealed conspicuous pathological changes associated with GVHD including emaciation, hepatic lesions, lymphoid atrophy, colitis, and diarrhea. Histologic examinations were performed on select animals.

Results

Inhibition of PHA Mitogenesis with Anti-Thy-1.2-Ricin. To determine whether antibody-ricin hybrid could inhibit T cell mitogenic responses, BALB/c splenocytes were

cultured in the presence of a stimulatory dose of PHA. (Fig. 1). Proliferative activity was measured by tritiated thymidine incorporation after 3 d. Activity was increased 48-fold (184,427 cpm) compared with cultures that were incubated in the absence of PHA (3,823 cpm) (see legend to Fig. 1). When 0.2 or 0.5 μg anti-Thy-1.2-ricin was added to mitogen-stimulated cultures in the presence of lactose, which competitively blocks galactose receptors on the hybrid molecule, PHA responses were reduced 89 and 94%, respectively, compared with the level of activity in control cultures (BALB/c splenocytes in the presence of mitogen). 1 μg/ml hybrid reduced activity even further. In contrast, when BALB/c splenocytes were cultured in the presence of the B lymphocyte-dependent mitogen LPS, 0.2 μg/ml and 0.5 μg/ml hybrid inhibited mitogenesis only 29 and 30% compared with control cultures. 1 μg/ml treatment had a much greater toxic effect. No protection was measured against the toxicity of hybrid when cells were incubated in the presence of mitogen, but without lactose in the culture system. Those concentrations of hybrid plus lactose which inhibited PHA-induced responses of BALB/c splenocytes had no effect on AKR splenocytes, which do not express Thy-1.2 antigen on their surfaces (not shown).

Protection of Allogeneic Irradiated Recipients from Lethal GVHD by Pretreatment of Donor Cells with Anti-Thy-1.2-Ricin. To determine whether GVHD-causing T cells could be eliminated from donor grafts with the anti-Thy-1.2-ricin hybrid before bone marrow transplantation, C57BL/6 mice were conditioned with 900 rad total-body irradiation (TBI). Irradiated recipients (n = 12) given BALB/c BMS (Allo-BMS) developed GVHD and showed a high (>90%) mortality rate (Fig. 2). These animals had engrafted before their death (not shown). Survival was significantly improved (91%) in recipients (n = 11) given donor BMS treated with antibody-ricin conjugate in the presence of lactose before injection. Surviving mice appeared healthy and showed no evidence of GVHD or subclinical GVHD on autopsy. Some degree of protection was conferred by treatment of donor BMS grafts with antibody alone (n = 18); however, as previously reported (3), this protection was only transient as recipients eventually

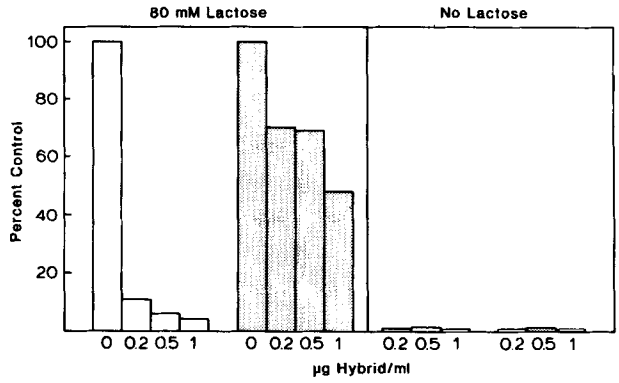

FIG. 1. BALB/c splenocytes were cultured in vitro in the presence of mitogens PHA (□) or LPS (▨). Varying amounts of αThy-1.2-ricin hybrid were added at the initiation of cultures, both with and without lactose. After 3 d cells were pulsed with tritiated thymidine, harvested, and counted by standard methods. Untreated cells responded vigorously to mitogens as follows: BALB/c cells + PHA = 184,427 cpm; BALB/c cells + LPS = 32,918 cpm; BALB/c cells alone = 3,823 cpm. Standard deviations of mean triplicate samples did not exceed 17%. Data are shown as percent control response in which the response of treated (αThy-1.2-ricin) cells is compared with untreated controls.

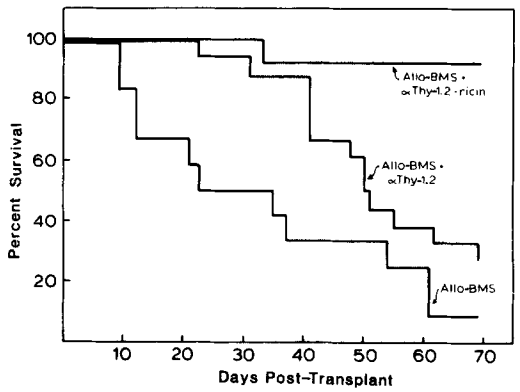

FIG. 2. A group of C57BL/6 mice were conditioned with 900 rad TBI and given Allo-BMS. Two other groups of recipients were given donor cells pretreated with 0.5 μg/ml anti-Thy-1.2-ricin plus lactose (Allo-BMS + αThy-1.2-ricin) or 0.5 μg/ml monoclonal anti-Thy-1.2 antibody alone (Allo-BMS + αThy-1.2). Calculation of survival results and statistical analysis have been reported elsewhere (3). χ-square analysis, which compared values over the entire length of the actuarial curve, showed that group Allo-BMS + αThy-1.2-ricin differed significantly from groups Allo-BMS and Allo-BMS + αThy-1.2 ($P < 0.001$). Also, group Allo-BMS + αThy-1.2 differed significantly from group Allo-BMS ($P < 0.003$). Group Allo-BMS contained 12 members; Allo-BMS + αThy-1.2, 18 members; and Allo-BMS + αThy-1.2-ricin, 11 members.

developed GVHD and survival was <28% by day 70. Control animals given grafts pretreated with 200 mM lactose alone or anti-Thy-1.1-ricin plus 200 mM lactose were not protected from GVHD (not shown). As further control, a group of irradiated C57BL/6 recipients were given syngeneic BMS cells. All of these mice remained alive, suggesting that deaths were not attributable to the toxicity of the radiation protocol (not shown). The data presented in Fig. 2 are representative of an experiment performed three times. To determine whether anti-Thy-1.2-ricin pretreatment resulted in engraftment, recipients from Fig. 2 were serotyped. A high percentage of donor-type (H-2^d) cells (a mean of 80%) were in the peripheral blood of these C57BL/6 mice 50–60 d after transplant.

Discussion

We have used a previously described model (3) in which the injection of donor bone marrow cells and splenocytes induces severe GVHD across major H-2 barriers. With death as a measured endpoint, elimination of immunocompetent T cells from donor grafts with monoclonal antibody plus complement protects irradiated recipients. Unfortunately, difficulties with complement sources and standardization preclude large-scale clinical use of this approach. In these studies we have circumvented the need for complement by synthesizing a cell type specific toxin made by linking ricin to anti-Thy-1.2 monoclonal antibody and then blocking the ricin galactose-binding site with exogenous lactose. This conjugate was effective in inhibiting protein synthesis of Thy-1.2-bearing EL4 tumor cells as reported (5). Moreover, the hybrid effectively eliminated the proliferative response of splenic T lymphocytes induced by T cell specific mitogen stimulation (Fig. 1). Although the conjugate did inhibit a B cell mitogen-induced response to some degree (≅29%), this could be attributed to the fact that antibody-ricin was left in the culture system during the entire duration of the assay and/or that a T cell mitogenic contamination was present in the LPS

preparation. Nonspecific stem cell toxicity was not encountered when donor cells were treated with antibody-ricin before transplant because recipients engrafted and displayed a high percentage of donor cells (>80%) in their peripheral blood.

Interestingly, in our transplant experiments it is apparently not necessary to eliminate all splenic T cells for successful therapy, as we have observed (8) that TBI-conditioned C57BL/6 mice given BALB/c marrow containing 1% T lymphocytes results in 60% survivors at 100 d post-transplant. Furthermore, Korngold and Sprent (9) showed in a system in which lethal GVHD could be induced across minor H-2 barriers, that by reducing the number of immunocompetent T cells present in the donor graft to 3×10^4, 20% survivors remained at 80 d post-transplant. Thus, during pretreatment, it may only be necessary to reduce the number of surviving T cells to 10^3 or 10^4 to achieve successful therapy in the mouse. Previous studies (10) on the ability of an anti-Thy-1.2 ricin conjugate to kill Thy-1.2-positive cells show that this range is achievable without severely depleting Thy-1.2 negative cells.

The hybrid used in these studies contains the ricin B chain to facilitate the entry of ricin A chain into the cytosol compartment. This is a necessary condition, as the affinity of the antibody used in the hybrid is only 10^6 M^{-1} measured with EL-4 cells at 25°C using ^{125}I-anti-Thy-1.2 (unpublished data). We have shown that the presence of the ricin B chain enhances the entry rate of ricin A chain into the cytosol and leads to more rapid killing and a lower fraction of survivors (10). Entry rate is also proportional to the number of occupied receptors. Consequently, hybrids made with relatively low affinity antibody, as in the present case, require the ricin B chain to achieve the necessary entry rate and low fraction of cell survivors. These considerations indicate that future anti-T cell hybrids of much greater selectivity can be constructed.

Others have used preparations of monoclonal antibodies conjugated directly to ricin A chain (11), circumventing the need for addition of exogenous lactose. Such a system would be valuable in certain cases where direct therapy with the hybrid was requisite, but at the present time a superiority of an A chain-antibody conjugate over an intact ricin has not been established. A direct comparison of Thy-1.1 antibody linked to intact ricin and antibody-linked A chain in vitro shows that both hybrids are effective, but that the intact ricin hybrid can have a faster rate of cell killing (10).

In this report, we have shown that monoclonal antibodies conjugated to intact ricin can be used to pretreat donor cells to specifically eliminate GVHD-causing cells. Such conjugates may serve as unique and powerful tools to control GVHD in human transplantation because of the simplicity of the treatment protocol. Furthermore, because the cells are incubated with hybrid and washed before injection, the risk of toxic effects against host tissue is minimized. Together, these studies suggest that the conjugation of ricin to antihuman T cell antibodies may provide useful tools for treatment of donor bone marrow in vitro before transplantation, permitting transplantation across major histocompatibility barriers.

Summary

A new method has been devised to eliminate T cells from murine bone marrow grafts across major histocompatibility barriers and thus prevent graft-vs.-host disease (GVHD). The method utilizes a monoclonal antibody directed at the Thy-1.2 antigen but is complement independent. To make anti-Thy-1.2 toxic, the antibody is covalently linked to the toxin ricin. Ricin ordinarily binds, enters, and kills cells through

receptors containing galactose. The hybrid protein, anti-Thy-1.2-ricin, can enter and kill cells via the Thy-1.2 receptor. In the presence of lactose the usual entry route for ricin is largely blocked and the hybrid is shown to be a highly selective reagent that is T cell specific in its inhibition of mitogen-stimulated splenocytes.

We have used a model of severe and fatal GVHD where BALB/c splenocytes and bone marrow cells are given to irradiated C57BL/6 recipients. Over 90% of these mice die by day 70, exhibiting signs of GVHD. When donor cells are pretreated with 0.5 μg/ml of anti-Thy-1.2-ricin plus 200 mM lactose before injection, 10 of 11 animals survive through day 70 without signs of GVHD. These studies demonstrate that ricin linked to monoclonal antibodies may have utility related to the prevention of GVHD in human bone marrow transplantation.

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References

1. Tyan, M. E. 1973. Modification of severe graft-versus-host-disease with anti-sera to the theta antigen or to whole serum. *Transplantation (Baltimore).* **15:**601.
2. Rodt, H. V., S. Theirfelder, and M. Eulitz. 1974. Anti-lymphocytic antibodies and marrow transplantation. III. Effect of heterologous anti-brain antibodies on acute secondary disease in mice. *Eur. J. Immunol.* **4:**25.
3. Vallera, D. A., C. Soderling, G. Carlson, and J. Kersey. 1981. Bone marrow transplantation across major histocompatibility barriers in mice. Effect of elimination of T cells from donor grafts by treatment with monoclonal anti-Thy-1.2 plus complement or antibody alone. *Transplantation (Baltimore).* **31:**218.
4. Vallera, D. A., C. C. B. Soderling, and J. H. Kersey. 1982. Bone marrow transplantation across major H-2 barriers. III. Treatment of donor graft with monoclonal antibodies directed against Lyt determinants. *J. Immunol.* **128:**871.
5. Youle, R. J., and D. M. Neville, Jr. 1980. Anti-Thy-1.2 monoclonal antibody linked to ricin is a potent cell type specific toxin. *Proc. Natl. Acad. Sci. U. S. A.* **77:**5483.
6. Ledbetter, J. A., and L. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse differentiation antigens. *Immunol. Rev.* **47:**63.
7. Vallera, D. A., C. Soderling, G. Carlson, and J. Kersey. 1981. Bone marrow transplantation across major histocompatibility barriers in mice. II. T cell requirement for engraftment in TLI-conditioned recipient. *Transplantation (Baltimore).* In press.
8. Vallera, D. A., A. Filipovich, C. C. B. Soderling, and J. H. Kersey. 1982. Bone marrow transplantation across major histocompatibility barriers. IV. GVHD in TLI-conditioned mice. *Clin. Immunol. Immunopathol.* In press.
9. Korngold, R., and J. Sprent. 1978. Lethal graft-versus-host-disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. *J. Exp. Med.* **148:**1687.
10. Neville, D. M., and R. Youle. 1981. Monoclonal antibody-ricin or antibody-ricin A chain hybrids: kinetic analysis of cell killing for tumor therapy. *Immunol. Rev.* In press.
11. Krolick, K. A., P. Isakson, J. Uhr, and E. Vitetta. 1980. Selective killing of normal or neoplastic B cells by antibodies coupled to the A chain of ricin. *Proc. Natl. Acad. Sci. U. S. A.* **77:**5419.